Part II. Challenges and efforts towards social implementation (including regulatory considerations)

15:40-16:40 Progress towards social implementation in Japan

1. Molecular analysis of novel crops produced by genome editing for application on commercial use

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Molecular analysis of novel crops produced by genome editing for application on commercial use

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Artificial restriction enzymes, such as CRISPR / Cas9 or TALEN, allow for accurate cleavage of specific target sequences, and it has become possible to arbitrarily induce gene modification (deletion, substitution or insertion) to the targeted domain on a genome (genome editing). By applying the genome editing to plant breeding, it would be possible to arbitrarily modify an endogenous gene that affects various traits, and thereby develop new innovative varieties in a short period.

Most genome editing in many plant species, foreign genes such as CRISPR/Cas9 or TALEN are introduced at first, accordingly those plants are regulated as a genetically modified organism (GMO) by the Cartagena domestic law (http://www.biodic.go.jp/bch/english/law.html) in Japan. Currently, various discussions are being conducted worldwide concerning the handling of null segregants in which foreign genes have been removed from those GM plants. LMO (living modified organism) are defined in Cartagena domestic law, as following.

> GMO and LMO have the same meaning in the present condition.
> 'LMO (living modified organism) shall mean an organism that possesses nucleic acid, or a replicated product thereof, obtained through use of the any of the following technologies.'

In Argentina, null segregants are not regulated as GM plants (Lema *et al.* 2015). Even in Japan, null segregants may not be regulated as GM plants from the provisions of Cartagena domestic law. Then, how can we prove a null segregant? Several techniques such as Southern blot analysis, PCR, Next Generation Sequencer (NGS), tiling array and others will be used for detection of introduced genes. However, since each method has advantages and disadvantages and it is necessary to select an appropriate method while taking into consideration the purposes and cost, other factors.

Here, I introduce application of these analysis methods for proof of null segregant.

Molecular analysis of novel crops produced by genome editing for application on commercial use

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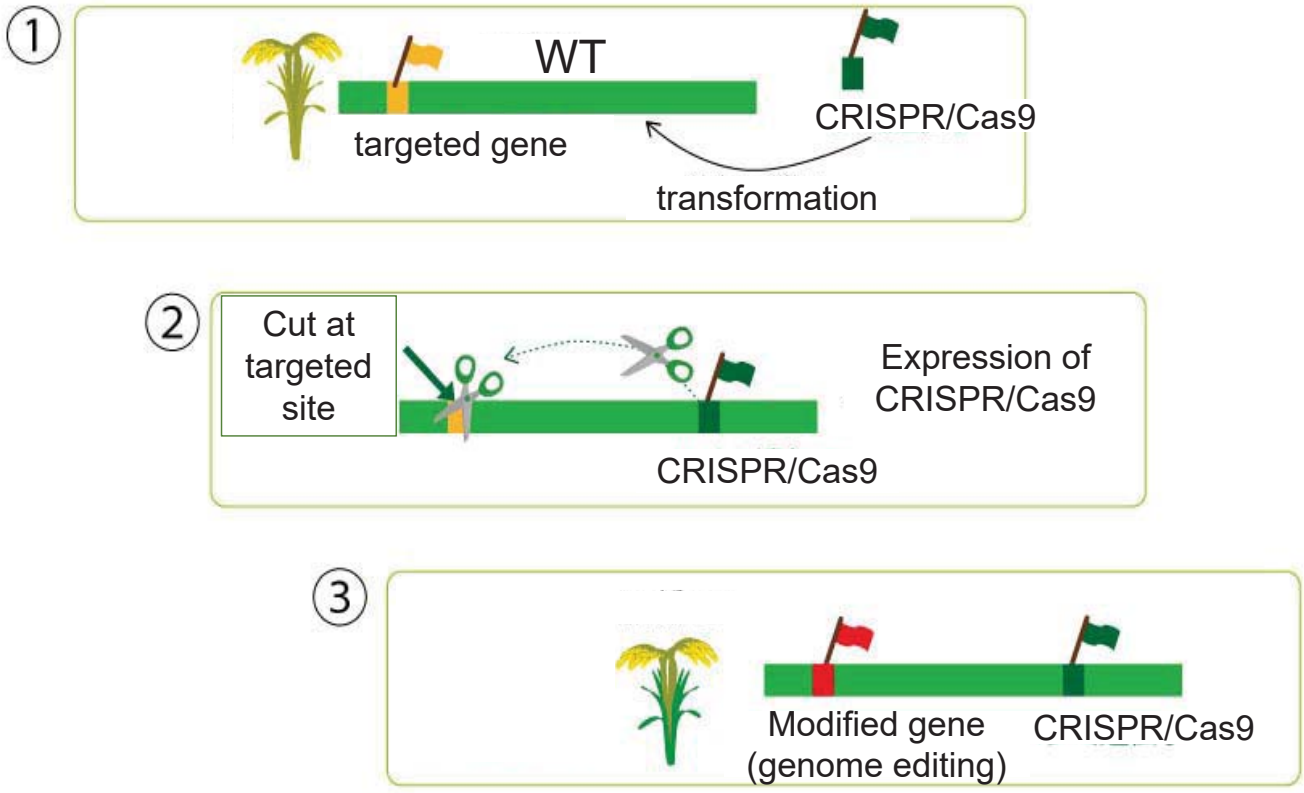
Novel crops will be produced by genome editing.

Are these novel crops GM crops or not?

Genome editing in plant



Modified original material by 'Life and BioPlaza21'

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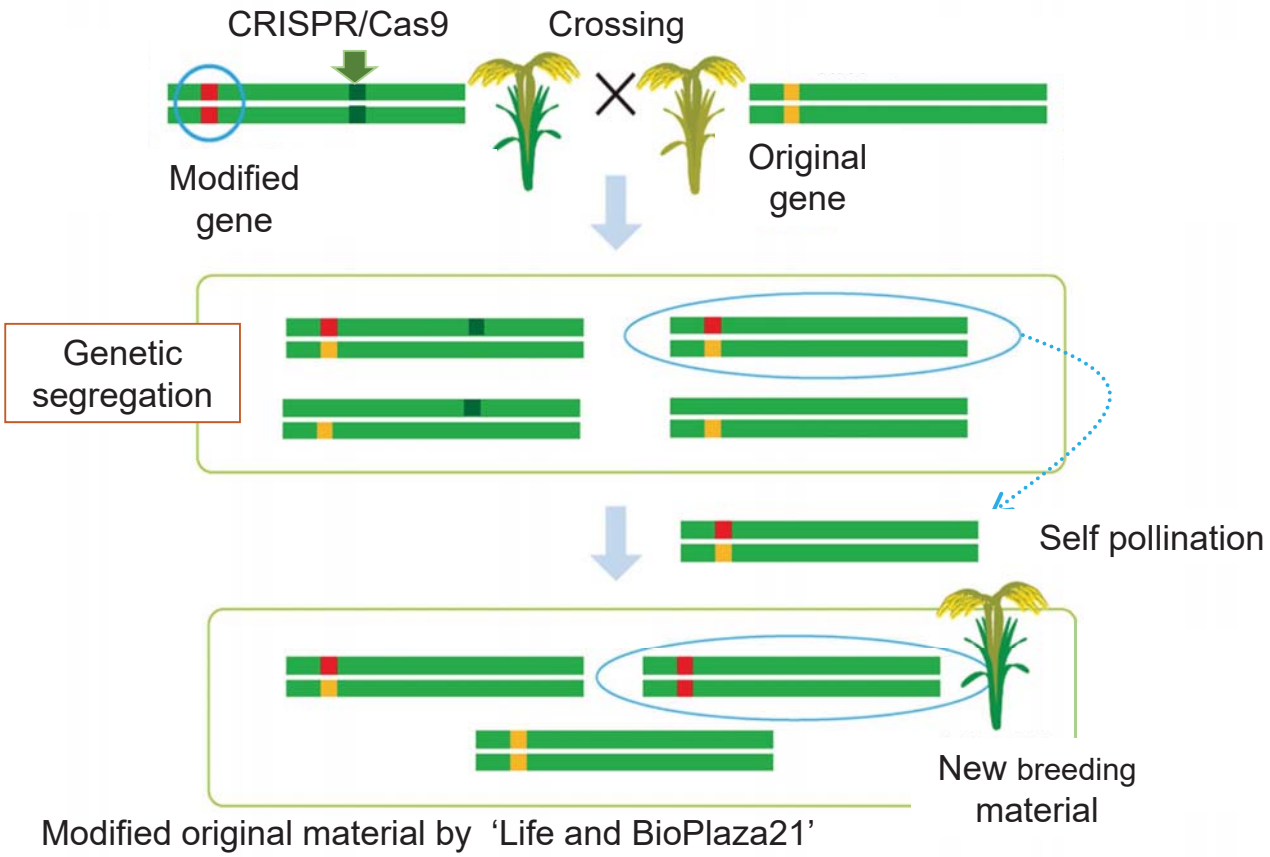
Production of null segregant



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Cartagena domestic Law

Definition of living modified organism

In this Act, “living modified organism” shall mean an organism that possesses nucleic acid, or a replicated product thereof, obtained through use of the any of the following technologies.

(i) Those technologies as stipulated in the ordinance of the competent ministries, for the processing of nucleic acid extracellularly.

exemption

A. The nucleic acid of living organism belonging to the same species as that of the living organism which the cells originate from.

B. The nucleic acid of living organism belonging to the species that exchanges nucleic acid with the species of the living organism which the cells originate from in natural conditions.

(ii) Those technologies, as stipulated in the ordinance of the competent ministries, for fusing of the cells of organisms belonging to different taxonomical families.

Novel crops produced by genome editing
Is this novel crops GM crops or not?

If novel crops produced by genome editing do not have any foreign genes,
the crops may be non-GMO.

Then, how do we prove elimination of unintended short-DNA insertion from the introduced gene such as the Cas9 gene.

Comparison of analysis methods

Southern blot analysis
- The most common method
- Sensitivity is not sufficient to detect small DNA fragment (?).

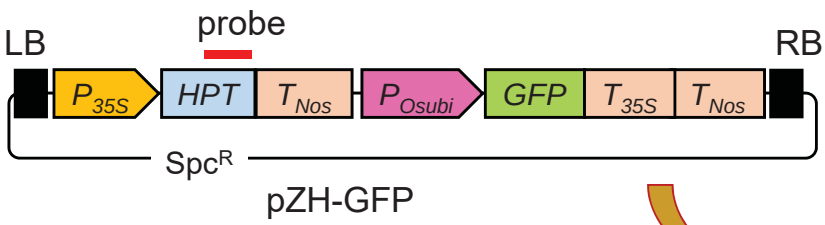
PCR: Polymerase chain reaction
- The most common method, the most sensitive if PCR works well.
- It is difficult to detect unintended small fragments derived from the introduced genes.

Tiling Array
- To detect all sequence of vector
- Sensitivity is not sufficient to detect small DNA fragment (?).

NGS: New Generation Sequencer
- High performance
- High precision reference and specialist for bioinformatics are essential.

Comparison of analysis methods

1) Production of transgenic rice as standard



pZH-GFP

Transformation by particle bombardment

2) Confirmation of detection sensitivity and accuracy

Southern blot analysis
PCR: Polymerase chain reaction
Tiling Array
NGS: New Generation Sequencer

Schematic diagrams for detection of unintended short DNA-insert derived from a recombinant DNA by Southern blot analysis

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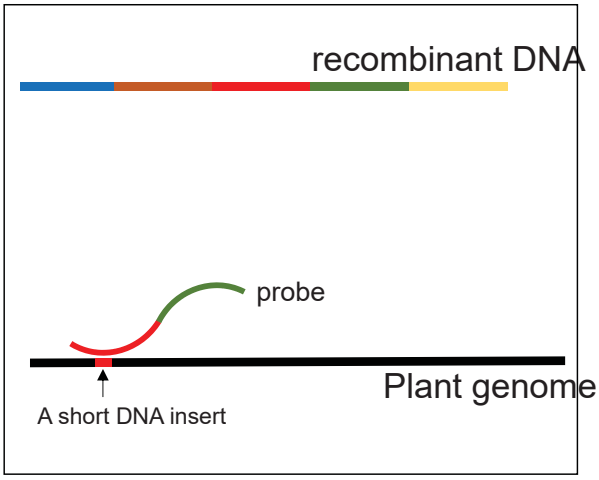
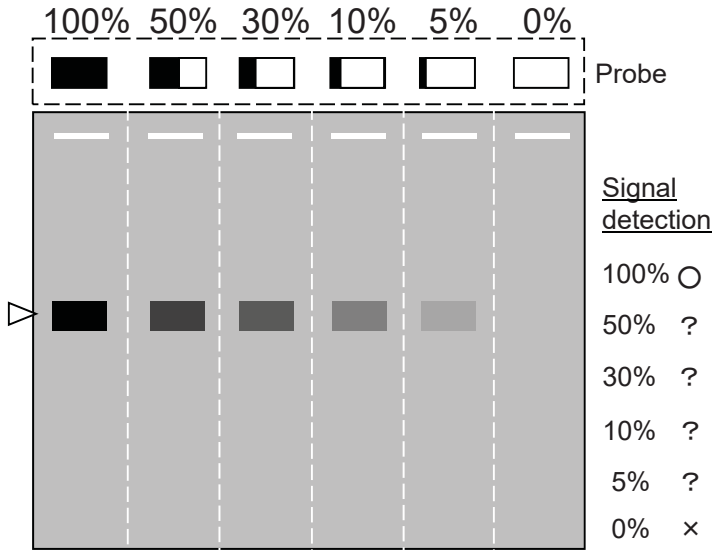
A schematic diagram of the model study

Endogenous single copy gene ・・

Dummy sequence ・・・・・・・

Image for detection of short rDNA(s)





Evaluation of the length limit of detection (1)

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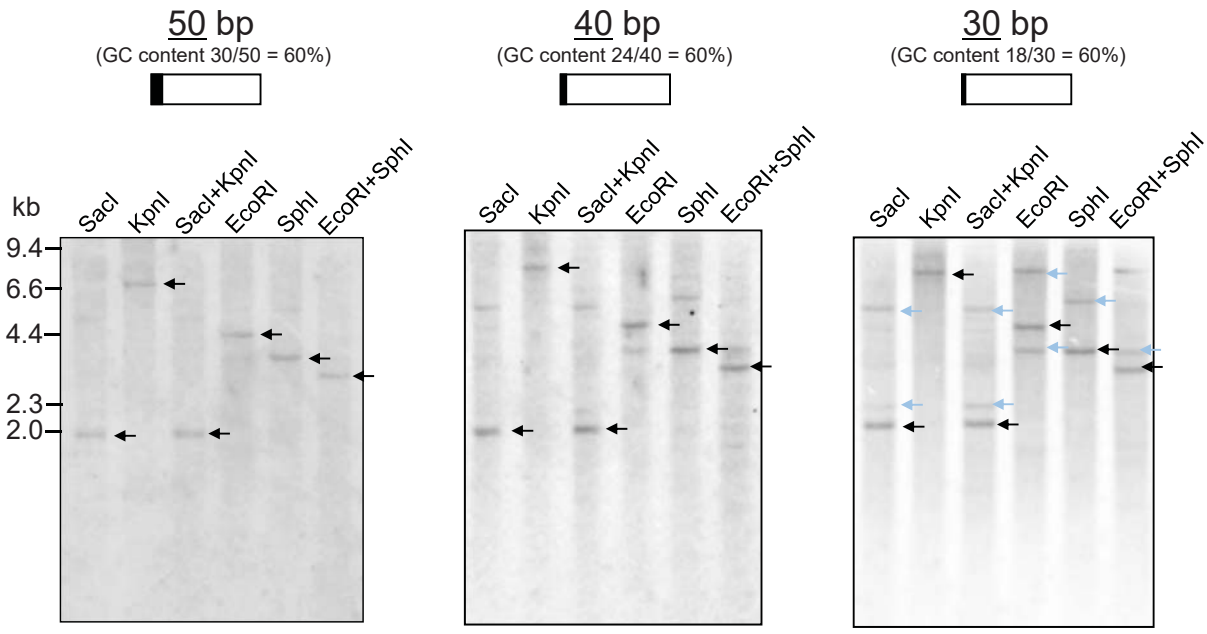
Phospholipase D

Hygromycin phosphotransferase



Evaluation of the length limit of detection (2)

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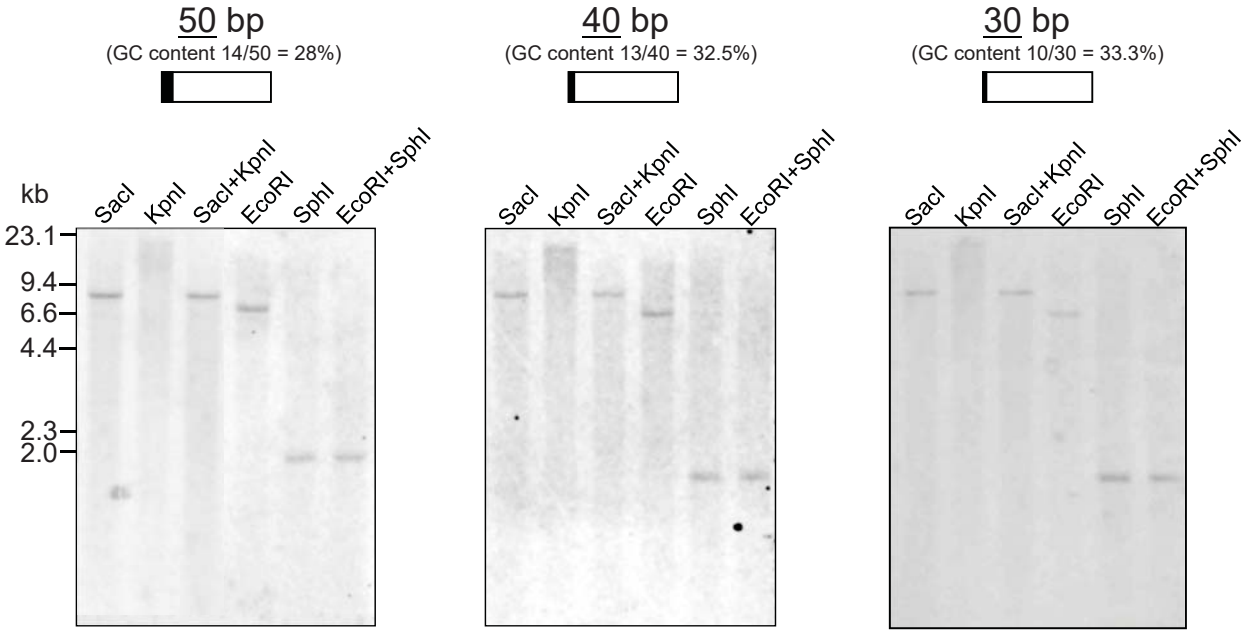
Sucrose phosphate synthase

Cauliflower mosaic virus 35S promoter



Array Comparative Genomic Hybridization

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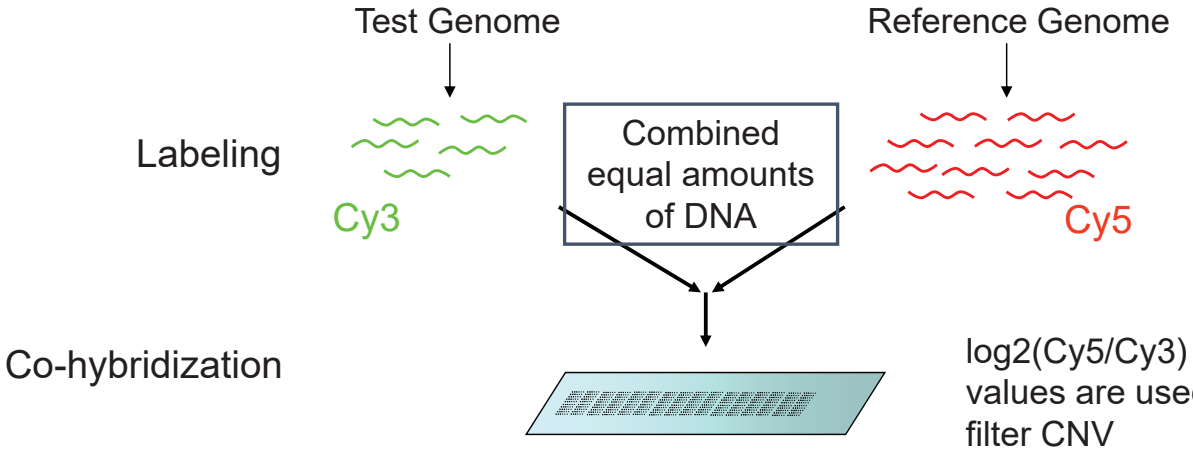
a high resolution genome analysis technique used to detect DNA

copy number variations (CNV)

→ target : whole genomes, loci of interest, vector sequences

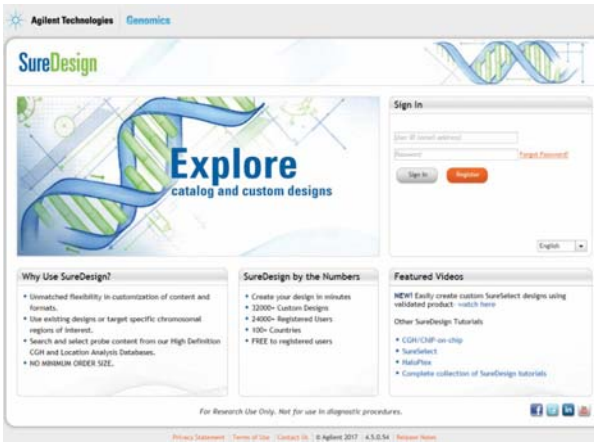
→ cheaper than whole-genome sequencing

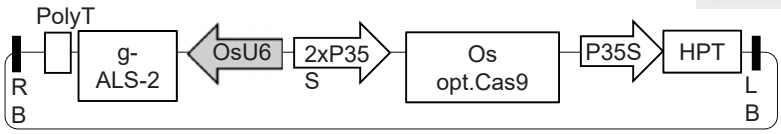


Custom-made tiling array

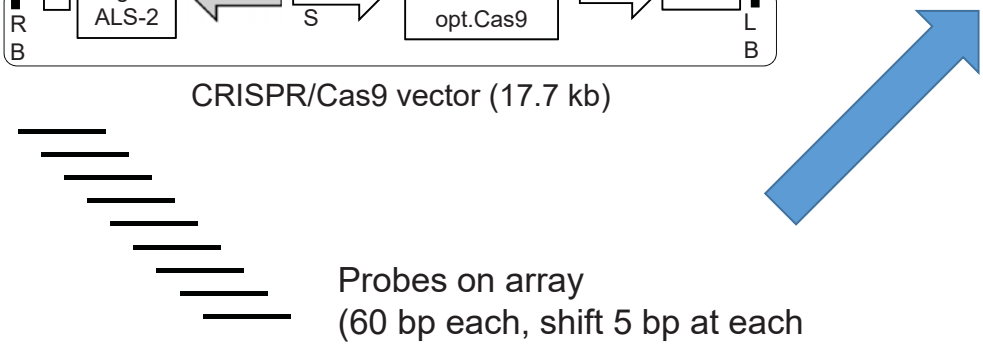
Agilent's online array design application

SureDesign
(https://earray.chem.agilent.com/suredesign/)





CRISPR/Cas9 vector (17.7 kb)



Probes on array
(60 bp each, shift 5 bp at each probe)

Comparison of NGS data mapping programs

Programs	Version	Reference
Bowtie2	2.2.5	Langmead B, Salzberg S. (2012) Fast gapped-read alignment with Bowtie 2. Nature Methods, 9:357-359.
BWA-MEM	0.7.12	Li H. and Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics, 25:1754-60.
GSNAP	2015-06-23	Wu TD and Nacu S. (2010) Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics, 26:873-881
Novoalign	V3.02.13	http://www.novocraft.com/
SMALT	v0.7.6	https://www.sanger.ac.uk/resources/software/smalt/

Comparison of NGS data mapping programs

	Bowtie2	BWA-MEM	GSNAP	Novoalign	SMALT
Bowtie2	3766	3354	3559	3342	3234
	-	(0.891)	**(0.945)**	(0.887)	(0.859)
BWA-MEM	3354	5495	5193	5127	4520
	(0.610)	-	**(0.945)**	**(0.933)**	(0.823)
GSNAP	3559	5193	6407	5109	4495
	(0.555)	(0.811)	-	(0.797)	(0.702)
Novoalign	3342	5127	5109	5377	4490
	(0.622)	**(0.954)**	**(0.950)**	-	(0.835)
SMALT	3234	4520	4495	4490	4716
	(0.686)	**(0.958)**	**(0.953)**	**(0.952)**	-

- The results of BWA-MEM and Novoalign are relatively similar.
- SMALT's results are highly similar to the others, but found less.
- GSNAP found more, but perhaps many of them are false positives.
- Bowtie2 found less. Plus, it is less sensitive than the others.

- The NGS technologies are powerful, but the mapping of NGS data is difficult. The results of different programs vary.
- We need a simple method that can produce consistent, stable data.
- In light of the regulations of the use of GMO, we do not necessarily detect all the nucleotide changes in the genome.

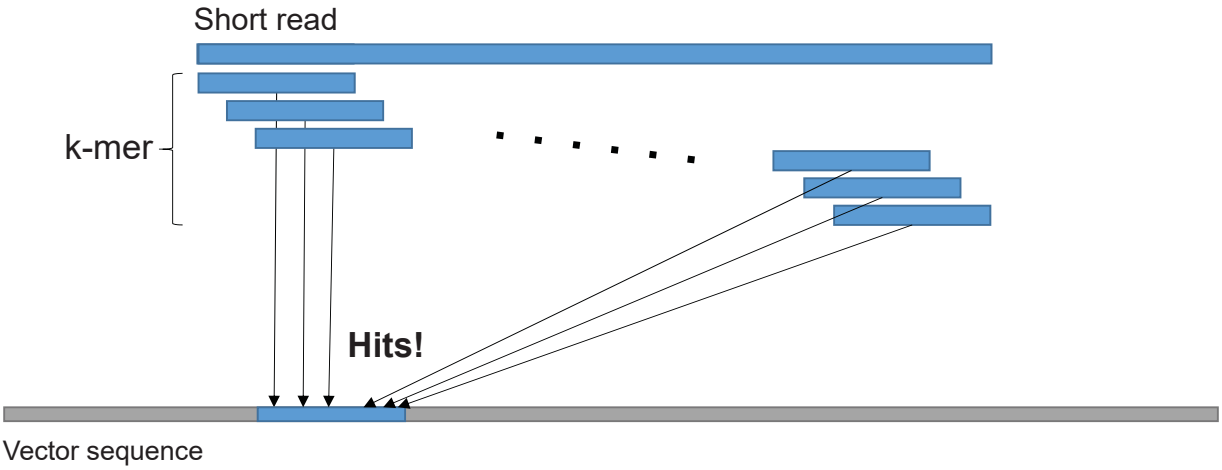
Detection of external DNA sequences by NGS

Here we focus on the detection of external DNA sequences that remain in the genome of a sample treated by genome editing, etc.

The genomic DNA sequences can be determined by a massive sequencing technology, such as Illumina HiSeq.

Detection of external DNA by small fragment pattern matching

- Obtain short reads of NGS from a treated sample (genome editing, GM, etc)
- Extract k-mers, sequence fragments of length k, from the reads
- If DNA of a vector remains in the treated sample, there should be some k-mers that match the vector sequence.



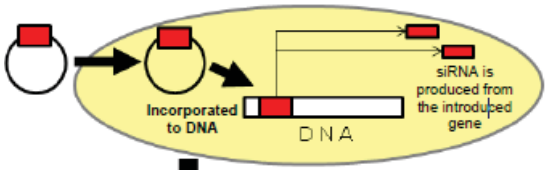
Considerations for practical application of NGS analysis

- To use this method, excellent informaticians are essential.
- In order to compensate for this, it is necessary to develop user-friendly analysis software.
- We should develop the analysis software and consider how to provide the analysis software.

- Open analytical software on the website and make it available by uploading data.
- Distribution of the software to researchers.

Proof of null segregant from other NPBT

- RNA-dependent DNA methylation
  siRNA-producing gene is introduced into plant geneme.

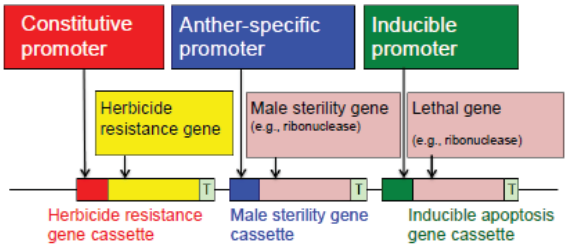


- Development of accelerated breeding of citrus by introduction of *CiFT* gene

  CiFT gene was introduced into trifoliate orange by *Agrobacterium* method, which was crossed with hyuganatsu (*Citrus tamurana*;cultivars). The hybrid seedling successfully flowered and fruited about 2 years after planting.

- Recurrent selection of autogamous crops such as rice

  Dominant male sterility crops are produced by integrated genes into autogamous crops and foreign genes are eliminated at final products.



COI Disclosure Information

Yutaka TABEI

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